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HRP-Antibody Conjugation Kit (CM51406x1 and CM51406x3) User Reference Guide

Contents

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Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

The information provided in this document and the methods included in this package are for information purposes only. CellMosaic provides no warranty of performance or suitability for the purpose described herein. The performance of labeling using this kit may be affected by many different variables, including but not limited to: purity and complexity of the antibody, differences in preparation techniques, operator abilities, and environmental conditions.

Sample data are provided for illustration and example purposes only and represent a small dataset used to verify kit performance in the CellMosaic laboratory. Information about the chemicals and reagents used in the kit are provided as necessary.

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Kit Components

This kit provides materials to perform HRP labeling of one (CM51406x1) or three (CM51406x3) antibody samples. The table lists the materials for one reaction.

⚠ Upon receipt, please remove **Box 1** and store in a freezer at or below -20°C. Store **Box 2** in a refrigerator at 2-8°C.

	Name	Part #	Quantity (CM51406x1)	Quantity (cm51406X3)	Storage condition
Box 1	C6 Maleimide Activated HRP (**red label**)	CM53214	1 unit	3 units	-20°C
	Reagent A (**cyan label**)	CM12101	1 unit	3 units	
	Reagent B Solution (**yellow label**)	CM12004.1	1 unit	3 units	
Box 2	Buffer A (**orange label**)	CM02001	4 mL	12 mL	2-8°C
	Buffer B (**indigo label**)	CM02005	10 mL	30 mL	
	PBS Buffer (**grey label**)	CM02013	4 mL	12 mL	
	Solution A (**green label**)	CM01003	4 mL	12 mL	
	Filter Device for Antibody	CM03CD050A	1	3	
	Filter Device for Conjugation	CM03CD010A	1	3	
	Desalting Column for Antibody	CM03SG05	1	3	
	Purification Column for Conjugates	CM03SD05	1	3	
	Airtight Syringe for Column	CM03SR2	1	3	
	1.5 mL Centrifuge Tubes	CM03CT2	2	6	
	Collection Tubes for Filter	CM03CT0	4	12	
	2.0 mL Centrifuge Tubes	CM03CT3	1	3	
User Material	IgG Antibody	N/A	NOT PROVIDED (User Supplied Material. 1 mg for each reaction)		

Safety Information

Warning: some of the chemicals used can be potentially hazardous and can cause injury or illness. Please read and understand the Material Safety Data Sheets (MSDS) available at CellMosaic.com before you store, handle, or use any of the materials.

Labeling Chemistry

The kit is designed to work with antibody IgG. The user supplies its own unmodified IgG. Using the kit components, the user converts the antibody to a thiol-antibody, followed by reaction of the thiol-antibody with activated HRP to generate the HRP-antibody conjugates. The combination of filtration and scavenger type purification steps typically provides the resulting HRP-antibody at greater than 90% purity.

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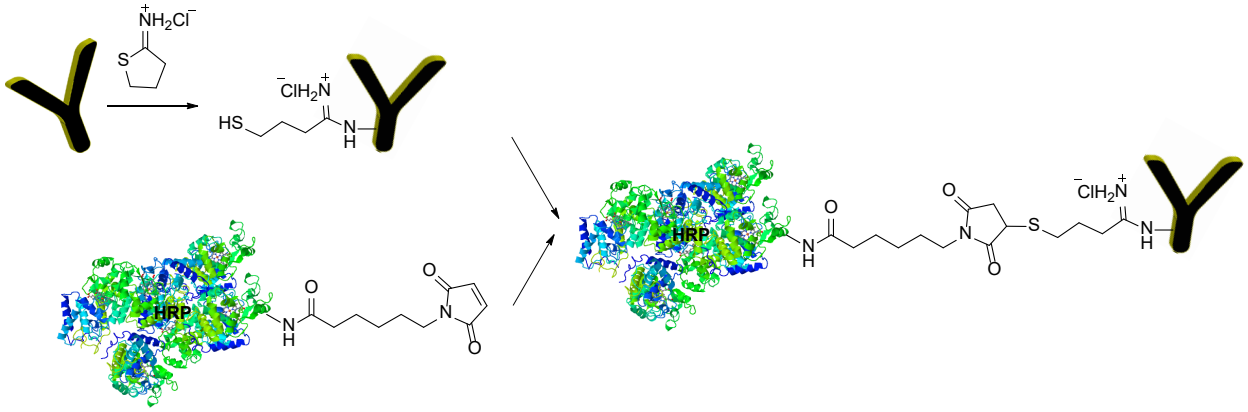

Scheme 1: Synthetic route to HRP–antibody conjugate.

Key features of this HRP antibody conjugation kit:

- High quality maleimide activated HRP for the conjugation: >99% purity and >200 units/mg protein activity
- Offers a convenient way to prepare HRP–antibody conjugate with heterobifunctional crosslinking reagents
    - Better control over the conjugation process
    - Limit self-coupling and polymerization that often encountered when EDC or homobifunctional crosslinkers are used
- Target average 2-4 HRP per antibody for a typical labeling
- Free of or less than 10% of unreacted HRP and antibody
- All reagents included, from preparation to purification
- Options to choose tailored services at CellMosaic after conjugation:
    - HPLC analysis of the sample
    - HPLC purification to remove any residual unreacted HRP and/or antibody

Requirement for antibody (IgG):
1. Preferably, the antibody should be >90% pure by gel electrophoresis
2. Total amount: 1 mg protein content as measured by UV. Note: the accuracy of your protein amount is the single most important factor to obtaining optimized loading. Please refer to the section Other Considerations in this manual to measure the protein content.

Potential interfering compounds for labeling and conjugation reactions: Thiols (e.g., DTT) and mercaptoethanol

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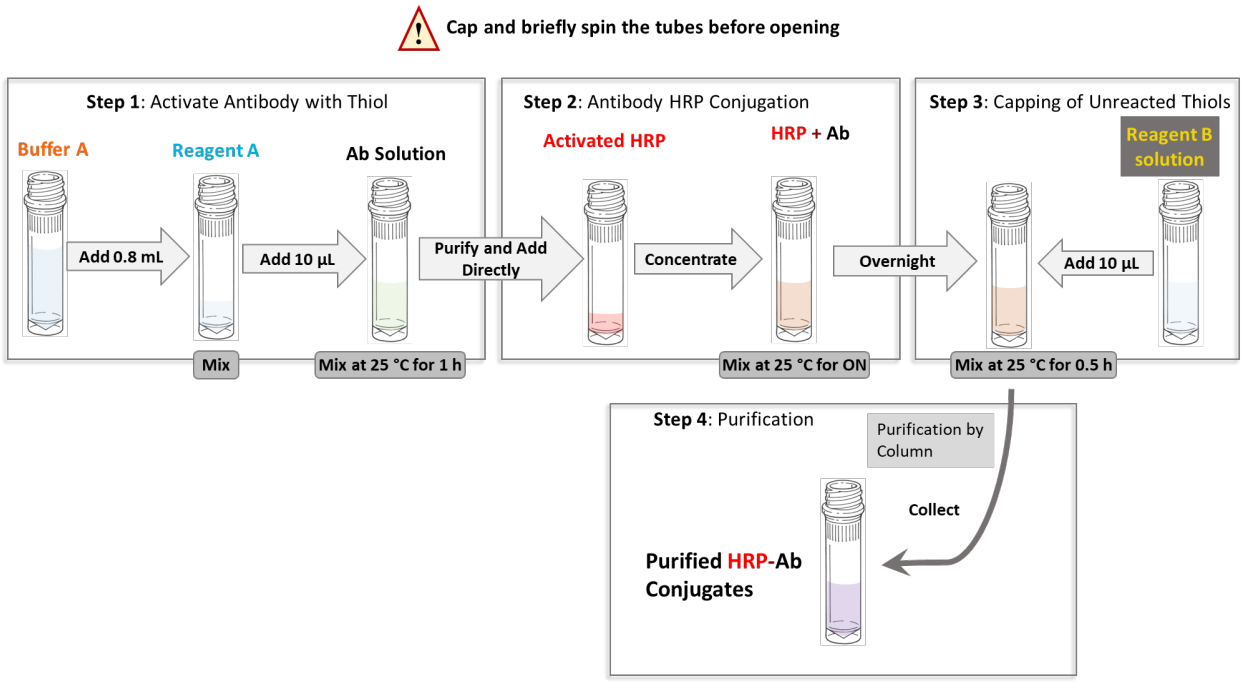
Protocol



Scheme 2. Schematic diagram of the workflow for preparing HRP–antibody conjugates

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated, e.g., Eppendorf 5417R)
- Pipettes and tips
- Timer
- Incubator or shaker set at 25°C (room temperature between 20–27°C is acceptable)
- Support stand, lab frame, or any support rod for desalting column
- Flask
- Personal protection equipment (lab coat, safety glasses, and chemical-resistant nitrile gloves)
- UV spectrophotometer (optional)

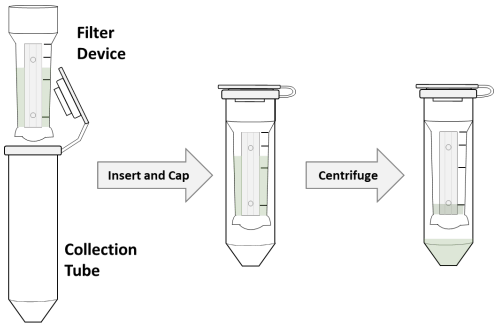
2. Preparation of Antibody Samples for Conjugation

Items needed: Antibody (user supplied), Filter Devices for Antibody (CM03CD050A), Collection Tubes for Filter (CM03CT0), Buffer A (CM02001, orange label), 1.5 mL Centrifuge Tubes (CM03CT2), Clean Centrifuge Tubes (not provided in the kit).

Total amount of antibody used for the conjugation is 1 mg (protein content measured by UV).

A1. Insert the **Filter Device for Antibody** (CM03CD050A) into one of the provided **Collection Tubes for Filter** (microcentrifuge tube with the cap attached). Perform the step based on the following conditions.

- ✓ If your antibody is supplied as a lyophilized solid, dissolve the antibody in 500 µL of deionized water and then transfer the entire contents to the Filter Device.
- ✓ If your antibody is supplied in < 500 µL buffer, transfer your antibody sample to the Filter Device directly. Add **Buffer A** to make up the total volume to 500 µL and cap it.
- ✓ If the volume of your antibody sample is >500 µL, add up to 500 µL of sample to the Filter Device. Repeat Step **A1-A4** until all of the antibody sample goes into the Filter Device. Move on to Step **A2**. Add **Buffer A** to make up the total volume to 500 µL for the last refill.



A2. Place the capped Filter Device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

A3. Spin the Filter Device at 14,000 x *g* for 8 minutes (preferably cooled to 4°C) to concentrate to < 50 µL. (Spin time depends on many factors. The typical spin time for a 500 µL sample in this Filter Device is approximately 6 to 10 minutes. The typical volume is ~35 µL after spinning for 8 minutes on an Eppendorf 5417R at 4°C).

A4. Remove the assembled device from the centrifuge and separate the Filter Device from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

A5. Insert the Filter Device back into the collection tube. Add 400 µL of **Buffer A** into the Filter Device. Spin the device at 14,000 x *g* to concentrate to < 50 µL. Remove the assembled device from the centrifuge. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

A6. Repeat Step **A5** one time.

A7. Transfer the concentrated sample from the Filter Device to one of provided **1.5 mL Centrifuge Tubes** (use a pipetman to measure the approximate volume of the concentrated sample).

A8. Wash the filter two times with 50 µL **Buffer A** and transfer the wash to the Collection Tube from Step **A7. (Note: Wash = Add buffer, aspirate with pipette 2-3 times.)**

A9. Add **Buffer A** to make up the total volume of the sample to ~200 µL and cap it.

A10. Vortex the combined antibody sample for 10 seconds and then centrifuge to ensure no liquid is in the cap.

3. Activate Antibody with Thiol (Step 1)

Items needed: Antibody solution from A10, Reagent A (CM12101, cyan label), Buffer A (CM02001, orange label).

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B1. Briefly spin the tube containing **Reagent A** (cyan label). Add 0.8 mL of **Buffer A** to the tube with **Reagent A**. Vortex for 30 seconds to 1 minute to dissolve the reagent.

Tip for solubility check: Check the bottom of the micro-centrifuge tube to see if the solution is clear and free of any solid residue.

B2. Transfer 10 µL Reagent A solution from Step **B1** to the 1.5 mL micro-centrifuge tube containing antibody solution from Step **A10**.

B3. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Mix the reaction mixture at RT for 1 hour (**Note:** While waiting for the reaction to complete, you need to move on to **Step C1** and equilibrate the column ahead of the purification).

Start Time: _____ End Time:_____

Tip for mixing: You can use a nutator, shaker, vortex, or incubator shaker for mixing. If you are using end to end nutating, make sure your centrifuge is capped properly. If you don't have any of this equipment, you can let the centrifuge tube sit at the bench with manual mixing by pipetting every 20 minutes.

4. Purification to Remove Excess Reagent A and Conjugation with HRP (Step 2)

Items needed: Desalting Column (CM03SG05), Filter Device for Conjugation (CM03CD010A), Collection Tubes for Filter (CM03CT0), Buffer B (CM02005, indigo label), C6 Maleimide Activated HRP (CM53214, red label), 1.5 mL Centrifuge Tubes (CM03CT2), Clean Centrifuge Tubes (not provided in the kit), Thiol Antibody Solution from **Step B3**.

C1. Securely attach the **Desalting Column** to a support stand, lab frame, or any support rod. Remove the top and bottom caps from the column and allow the excess liquid to flow through by gravity. Collect the liquid in a flask (waste).

C2. Add 2.5 mL of **Buffer B** (indigo label) and allow the buffer to completely enter the gel bed by gravity flow (waste).

C3. Repeat Step **C2** two times.

C4. Spin the thiol-modified antibody from Step **B3** to ensure there is no liquid in the cap before opening it. Add the entire antibody solution to the column. Allow the sample to enter the gel bed completely (waste).

C5. Add 290 µL of **Buffer B** and allow the buffer to completely enter the gel bed by gravity flow (waste)

C6. Place the tube containing 2 mg of **C6 Maleimide Activated HRP** (red label) under the column. Add 710 µL of **Buffer B** to the column. Collect the eluent by gravity and allow the buffer to enter the gel bed completely.

C7. Cap and vortex the tube from Step **C6** for 30 seconds to 1 minute to dissolve the HRP. Spin the reaction mixture to ensure there is no liquid in the cap before opening it.

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C8. Insert **Filter Device for Conjugation** (CM03CD010A) into one of the provided **Collection Tubes for Filter**. Transfer up to 500 µL of the reaction mixture from Step **C7** to the Filter Device. Place the capped Filter Device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the device at 14,000 x *g* for 5 minutes (preferably cooled to 4°C) to concentrate to < 150 µL. (The typical volume is ~120 µL after spinning for 6 minutes on an Eppendorf 5417R at 4°C).

C9. Transfer the rest of the reaction mixture from Step **C7** to the Filter Device. Washing the tube from Step **C7** with 100 to 150 µL of Buffer B. Then transfer the washing buffer to the Filter Device. Spin the device at 14,000 x *g* for 5 minutes (preferably cooled to 4°C) to concentrate to ≤150 µL.

C10. Transfer the concentrated sample from the Filter Device to one of the provided **1.5 mL Centrifuge tubes**.

C11. Wash the filter two times with 20 µL Buffer A and transfer the wash to the Collection Tube from **Step C10. (Note: Wash = Add buffer, aspirate with pipette 2-3 times.)**

C12. Add Buffer B to make up the total volume of the samples to 190 µL. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Mix the reaction mixture at RT for overnight (16 to 20 hours).

Start Time: _____ End Time:_____

5. Capping Unreacted Thiol Groups of Antibody (Step 3)

Items needed: Reagent B Solution (CM12004.1, yellow label), Conjugate Solution from **Step C12**.

D1. Briefly spin the tube containing **Reagent B Solution** (yellow label). Transfer 10 µL **Reagent B Solution** to the reaction mixture from Step **C12**.

D2. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Mix the reaction mixture at RT for 30 minutes to 1 hour.

Start Time: _____ End Time:_____

6. Purification to Remove Excess Reagent B and Unreacted HRP (Step 4)

Items needed: Purification Column for Conjugates (CM03SD05), Airtight Syring for Column (CM03SR2), Collection Tubes for Filter (CM03CT0), 2.0 mL Centrifuge Tube (CM03CT3), PBS Buffer (CM02013, grey label), Conjugate Solution from **Step D2**.

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CellMosaic's purification column has a unique design that will allow customers to do low-pressure column purification quickly.

Column Design: Column comprises five pieces (see left picture)

1. **Male Luer lock cap** (referred to as **top cap** in the protocol and is used during mixing/nutation).
2. **Column top with Luer attachment** (referred to as **column top** in the protocol). The Luer attachment is for a tight seal and attachment of the syringe for washing. The column top is removed for addition of buffer and stirring.
3. **Column body containing one polypropylene frit at the bottom** (referred to as **column** in the protocol).
4. **Top frit** (cover the resin, referred to as **frit** in the protocol).
5. **Bottom female Luer lock plug** (referred to as **bottom plug** and is used during mixing/nutation and storage).

Column Setup (see right picture): Securely attach the column to a support stand, lab frame, or any support rod and place a beaker or flask under the column for waste collection.

E1. Remove the purification column from the sealed plastic. Remove the **top cap** and unscrew the **column top**. Decant the PBS buffer from the **column** to the waste.

E2. Securely attach the column (see **column setup**). Make sure there is no residual liquid on the **frit** (note: You can use pipettor to remove any residual liquid on the top of the **frit**).

E3. Centrifuge to tube containing the conjugate solution from **Step D2** to ensure no liquid is in the cap before opening. Pipette the solution out and add directly onto the center of the **frit**.

Load, Wash, and Elute Setup (E4 to E7)

The column comes with a 5 mL airtight syringe that can be used to apply pressure to the column by pushing air through the column.

E4. Attach the column top to the Luer attachment. Draw 5 mL of air into the airtight syringe and attach the syringe to the column top. Slowly push the air through the column to get the liquid to elute **drop by drop** (*Do Not Overuse the Pressure and Dry the Resin*). **Allow only 6 drops of liquid to drain to the waste.**

E5. Remove the column top. Wash the centrifuge tube from **Step D2** with 200 µL of PBS buffer. Pipette the solution and add it directly onto the center of the **frit**. Repeat **Step E4** procedure to

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push the liquid out (*Do Not Overuse the Pressure and Dry the Resin*). **Allow only 6 drops of liquid to drain to the waste**.

E6. Remove the column top and fill the column with 1.0 mL of PBS buffer. Repeat **Step E4** procedure. Pay attention to the liquid line. Stop when the liquid is completely entered into the **frit**. **Allow up to 34 drops (Do not exceed 34 drops) of liquid to drain to the waste.**

E7. Remove the column top, fill the column with 1.2 mL of PBS buffer. Put a new 2.0 mL centrifuge tube under the column to collect the product. Pay attention to the liquid line. Stop when the liquid is completely entered into the **frit**. **Allow up to 40 drops of liquid (Do not exceed 40 drops) to drain to the centrifuge tube.** Label it as your product.

E8. Vortex the combined conjugates from **Step E7** for 30 seconds and then centrifuge to ensure no liquid is in the cap.

HRP-Antibody is Ready for Your Experiment

Tip: The approximate concentration of the conjugate is 0.2-0.6 mg/mL in PBS buffer. The number of HRP molecules per antibody is 2-4 on average.

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Other Considerations

1. Concentration Determination for IgG Antibody (Unlabeled)

The accuracy of the IgG amount is important for obtaining optimized loading in this protocol. The simplest assay method for determining IgG concentration in solution is to measure the absorbance of the IgG at 280 nm (UV range) ($A_{1\ mg/mL}$ = 1.4).

If your antibody comes with a buffer that has no UV absorbance at 280 nm, you can measure the UV absorbance prior to starting an experiment.

$$Concentration\ (mg/mL)\ of\ IgG = \frac{(A280)}{1.4}$$

If your antibody comes with a buffer that has UV absorbance at 280 nm, you can determine the concentration in **Step A9** after exchanging it with Buffer A and assuming **95%** recovery of the IgG after buffer exchange. Buffer A does not contain any substances that will interfere with the UV measurement at 280 nm.

$$Concentration\ (mg/mL)\ of\ Starting\ IgG = \frac{(A280)}{1.4 \times 0.95}$$

After calculating the total amount, follow the calculations in **Steps B10, C3**, **D9**, **E2**, **F5,** and **F6** to obtain the correct volumes to be added in each step.

2. Concentration Determination for Conjugate

To determine the concentration, dilute your HRP-antibody with 1 x PBS buffer. Measure the UV Absorbance of HRP-antibody at 403 nm (A_{403}) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (\mu M\ of\ HRP) = \frac{\mathrm{A403}\ \times 10}{\mathrm{L}\ \times 1.02}$$

$$Concentration\ (\mu M\ of\ Conjugate) = \frac{\mathrm{Concentration\ of\ HRP\ in\ \mu M}}{\mathrm{n}}$$

L: UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute HRP-antibody 5 to 10 times to get a good reading.

n: average number of HRP molecules per antibody. Use 2.0 if you do not have this data.

3. MW Calculation for Conjugate

Calculation of the MW of the conjugate:

MW = n x 44,000 + 150000

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Where n is the average number of HRP molecules per antibody. Use 2.0 if you do not have this data.

4. Analyze the Conjugate by HPLC

The purity of the conjugate can be analyzed by size exclusion chromatography (SEC). SEC separates the conjugates by apparent molecular weight (MW) or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes. However, the SEC profile may not be useful for calculating the actual loading. As both antibody and HRP are activated via surface amines, resulting in very heterogeneously distributed conjugates, you may get a very broad peak containing various degrees of HRP-loaded antibody.

CellMosaic offers two SEC standards (Product #: CM92004 and CM92005) for our customers to use with any SEC column. The CM92004 product sheet contains all the information and methodology you need to run an SEC HPLC analysis.

If you do not have access to an HPLC facility, you can send your sample to CellMosaic for analysis.

5. Recommended Storage Conditions

Depending on the stability of your antibody, HRP-Antibody conjugate solution is recommended to store at 2-8 °C and should be used as soon as possible. Some HRP–antibody conjugates may be stored at or below -20°C or lyophilized for long-term storage. Avoid repeated freeze-thaw cycles.

The stability of your conjugate may be different due to your antibody and should be checked by either HPLC or UV.

6. Sample Submission for HPLC Analysis

If you are submitting samples to CellMosaic for SEC analysis, please follow these instructions:

1) Go online: https://www.cellmosaic.com/hplc-analysis/, select SEC HPLC Analysis (Product# AS0023), choose the quantity (number of samples. Bulk discounts available for multiple samples) and submit the order. Alternatively, you can email info@cellmosaic.com for a quote and to place the order.
2) Dilute your un-conjugated antibody in PBS buffer to 1 mg/mL, and then transfer 50 µL of the diluted solution to a 500 µL micro-centrifuge tube. Label the vial properly.
3) Dilute your conjugated antibody in PBS buffer 4 times and transfer 50 µL of the diluted solution to a 500 µL micro-centrifuge tube. Label the vial properly.
4) Ship your samples with a cold pack for overnight delivery.